Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US05/005955

International filing date: 28 February 2005 (28.02.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/548,635
Filing date: 27 February 2004 (27.02.2004)

Date of receipt at the International Bureau: 23 March 2005 (23.03.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

1295758

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

March 14, 2005

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/548,635*
FILING DATE: *February 27, 2004*
RELATED PCT APPLICATION NUMBER: *PCT/US05/05955*

Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

PROVISIONAL APPLICATION FOR PATENT
COVER SHEET

Case No. **HITACHI.064PR**
Date: February 27, 2004

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

ATTENTION: PROVISIONAL PATENT APPLICATION

Sir:

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR § 1.53(c).

For: **MULTIPLEX DETECTION PROBES**

Name of Inventor: Taku Murakami
Residence Address: 280 Las Palmas
Irvine, California 92602

Enclosed are:

(X) Specification in 16 pages.

(X) 6 sheet(s) of drawings.

(X) A check in the amount of $160 to cover the filing fee is enclosed.

(X) A return prepaid postcard.

(X) The Commissioner is hereby authorized to charge any additional fees which may be required, now or in the future, or credit any overpayment to Account No. 11-1410.

Was this invention made by an agency of the United States Government or under a contract with an agency of the United States Government?

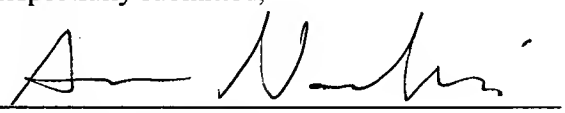
(X) No.

( ) Yes. The name of the U.S. Government agency and the Government contract number are:

(X) Please send correspondence to:

Arman H. Nadershahi
Knobbe, Martens, Olson & Bear, LLP
2040 Main Street, 14th Floor
Irvine, CA 92614

Respectfully submitted,



Arman H. Nadershahi
Registration No. 55,479
Customer No. 20,995
(949) 760-0404

H:\DOCS\AHN\AHN-1189.DOC
022504

Knobbe Martens Olson & Bear LLP
Intellectual Property Law

2040 Main Street
Fourteenth Floor
Irvine, CA 92614
Tel 949-760-0404
Fax 949-760-9502
www.kmob.com

Arman H. Nadershahi

MAIL STOP PROVISIONAL PATENT APPLICATION
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

Attorney Docket No.	:	HITACHI.064PR
Applicant(s)	:	Taku Murakami
For	:	MULTIPLEX DETECTION PROBES
Attorney	:	Arman H. Nadershahi
"Express Mail" Mailing Label No.	:	EV 307984639 US
Date of Deposit	:	February 27, 2004

I hereby certify that the accompanying

Transmittal letter; specification in 16 pages; 6 sheets of drawings; Check(s) for Filing Fee(s); Return Prepaid Postcard

are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and are addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Nelson Merida

H:\DOCS\AHN\AHN-1190.DOC
022504

MULTIPLEX DETECTION PROBES

Background of the Invention

Field of the Invention

[0001] The present invention relates generally to the field of chemical analysis. More particularly, it concerns tag reagents for sensitive, high throughput detection and analysis of target molecules.

Description of the Related Art

[0002] Chemical labels, otherwise known as tags or signal groups, are widely used in chemical analysis. Among the types of molecules used are radioactive atoms, fluorescent reagents, luminescent reagents, metal-containing compounds, electron-absorbing substances and light absorbing compounds. A number of different types of molecules have been used as tags that can be differentiated under mass spectrometry. Chemical signal groups can be combined with reactivity groups so that they might be covalently attached to the target, the substance being detected.

[0003] However, current detection probes do not adequately allow highly multiplex detection and analysis of molecules. Microarrays can analyze the expression profiles of thousands of genes, but researchers can typically handle only one or two samples on a single microarray chip or slide because their fluorescent or luminescent detection systems have very limited multiplex capability. In addition to the added costs caused by the necessary use of multiple chips or slides, the limited analytical capacity of existing methods makes it difficult to replicate microarray experiments and/or compare data among samples. Moreover, while many other applications and assays have been developed using microplate formats, the use of current multiplex methods and devices limit the number of the samples that can be used in each well. Using current methods and devices of multiplex analysis of molecules requires multiple wells and/or plates for higher throughput and reproducible data.

Summary of the Invention

[0004] Embodiments of the present invention to provide compositions and methods relating to the use of release tag compounds for detection and analysis of target

molecules, which increase signal intensity of molecular probes and allows for sensitive, high-throughput multiplex analysis. Liposome embodiments preferably contain a plurality mass tag molecules, ranging in number from 1 to 2 x 10^8 which provides stronger signaling and allows for highly sensitive multiplex analysis of molecules. Other embodiments can contain many orders of magnitude more mass tag molecules. For example, a solid particle having a diameter of 0.5 μm can contain up to 2 x 10^{10} mass tag molecules. Still other embodiments can have as high as possible depending the size of the embodiments and the density of the mass tag molecules, but ordinarily there are less than 1 x 10^{20}, and more preferably less than 1 x 10^{15} molecules.

[0005] Preferred embodiments comprise detection probes utilizing vesicles to retain multiple mass tag molecules, which are molecules with a specific molecular mass (or mobility) detectable by mass spectrometry, electrophoresis, chromatography or other analytical methods known to those skilled in the art. Embodiments include, but are not limited to, encapsulation vesicles, uni-lamellar vesicles, and multi-lamellar vesicles. The various vesicles preferably comprise liposomes, which preferably comprise a plurality of phospholipids. In certain embodiments, the mass tag molecules can be attached to the phospholipids themselves. Other molecules, such as cholesterol or other hydrophobic molecules, also can be entrapped in the lipid bilayer through hydrophobic interaction as mass tag molecules.

[0006] Further embodiments of the present invention comprise various carriers of mass tag molecules, including, but not limited to: emulsion, soluble beads, soluble capsules, and soluble porous beads.

[0007] Embodiments of the present invention can be used with various analytical methods and systems, including, but not limited to: hybridization assays, multiplex microarray assays, multiplex immunoassays, multiplex hybridization assays, multiplex CpG methylation assays, capillary assays, mass spectrometry, electrophoresis, and other analytical methods and systems known to those skilled in the art.

Brief Description of the Drawings

[0008] FIGs 1A-1C are schematic representations of various types of mass tag-containing vesicles.

[0009] FIG 2 is a graph showing hybridization assay results for various vesicle types.

[0010] FIG 3 is a schematic depiction of a multiplex microarray process with mass tag molecules.

[0011] FIG 4 is a schematic depiction of a multiplex immunoassay with mass tag molecules.

[0012] FIG 5 is a schematic depiction of a multiplex hybridization with mass tag molecules.

[0013] FIG 6 is a schematic depiction of a capillary assay with mass tag molecules.

Detailed Description of the Preferred Embodiment

[0014] Embodiments of the present invention comprise detection probes utilizing various forms of vesicles to retain multiple mass tag molecules. Mass tag molecules are disclosed in U.S. patent number 6,635,452, which is hereby incorporated in its entirety by reference. Mass tag molecules of the present invention comprise molecules with a specific molecular mass or mobility detectable by various analytical methods and systems including, but not limited to: hybridization assays, multiplex microarray assays, multiplex immunoassays, multiplex hybridization assays, multiplex CpG methylation assays, capillary assays, mass spectrometry, electrophoresis, and other analytical methods known to those skilled in the art.

[0015] The embodiment illustrated in FIG 1A shows a detection probe 20 comprising an encapsulation vesicle 22 having at least one mass tag molecule 24 preferably located within the vesicle 22. The vesicle preferably comprises at least one interaction site 26 on its surface. The vesicle encapsulates at least one mass tag molecule 24 with a specific molecular mass.

[0016] The embodiment illustrated in FIG 1B shows a detection probe 30 comprising a uni-lamellar vesicle 32. The membrane 38 of the vesicle 32 preferably comprises mass tag molecules 34 with a specific molecular mass. Further embodiments of

the uni-lamellar vesicles 32 comprise at least one mass tag molecule 34 preferably located within the uni-lamellar vesicle 32. The mass tags 34 located within the vesicle 32 are preferably the same type of mass tags 34 as those comprising the membrane 38. The vesicle preferably comprises at least one interaction site 36 on its surface.

[0017] FIG 1C shows another preferred embodiment of the present invention which comprises a detection probe 40, further comprising a multi-lamellar vesicle 42. The membrane 48 of the vesicle preferably comprises mass tag molecules 44 with a specific molecular mass. Further embodiments of the uni-lamellar vesicles 42 comprise at least one mass tag molecule 44 preferably located within the uni-lamellar vesicle 42. The vesicle 42 preferably encapsulates at least one smaller vesicle 49 which preferably contains the same type of mass tags 44 as those comprising the membrane 48. The mass tags 44 located within the vesicle 42 are preferably the same type of mass tags 44 as those comprising the membrane 48. The vesicle 42 preferably comprises at least one interaction site 46 on its surface.

[0018] Embodiments of the detection probes 20, 30, and 40 of FIGs 1A-1C utilize vesicles comprising liposomes, which can carry and release mass tag molecules. In order to release the mass tag molecules for detection, these carriers are preferably easily disrupted by physical stimulation, including but not limited to: heat, centrifugation, laser irradiation, sonication, electricity, evaporation, freeze-thaw process, or other methods known to those skilled in the art. Disruption may also be preferably achieved by chemical stimulation including, but not limited to: addition of organic solvent, detergent, acid, alkaline, enzyme, chaotropic reagents (urea, guanidium chloride, etc.), change of buffer, change of salt, change of concentration, change of pH, change of osmotic pressure, and other methods known to those skilled in the art.

[0019] In preferred embodiments, the probes 20, 30, and 40 have interaction sites 26, 36, and 46 on their outer surface comprising chemical residues, polynucleotides, proteins, peptides, carbohydrate or other small compounds known to those skilled of the art. In preferred embodiments, the molecules of the interaction sites 26, 36, and 46 are immobilized. The interaction sites 26, 36, and 46 of the probes 20, 30, and 40 can preferably be used to analyze various intermolecular interactions such as nucleotide-nucleotide interactions

(hybridization), antigen-antibody interactions (immunoassay), protein-protein interactions, small compound-protein interactions, small compound-cell interactions, and other interactions known to those skilled in the art.

[0020] As defined herein, the term "interactive site" refers to a group capable of reacting with the molecule whose presence is to be detected. For example, the interactive site may be a biomolecule capable of specific molecular recognition. Biomolecules capable of specific molecular recognition may typically be any molecule capable of specific binding interactions with unique molecules or classes of molecules, such as peptides, proteins, polynucleic acids, carbohydrate, and other chemical molecules, etc.

[0021] Thus, interactive sites disclosed herein for use with the disclosed methods encompass polypeptides and polynucleic acids. As used herein, polypeptides refer to molecules containing more than one amino acid (which include native and non-native amino acid monomers). Thus, polypeptides includes peptides comprising 2 or more amino acids; native proteins; enzymes; gene products; antibodies; protein conjugates; mutant or polymorphic polypeptides; post-translationally modified proteins; genetically engineered gene products including products of chemical synthesis, in vitro translation, cell-based expression systems, including fast evolution systems involving vector shuffling, random or directed mutagenesis, and peptide sequence randomization. In preferred embodiments polypeptides may be oligopeptides, antibodies, enzymes, receptors, regulatory proteins, nucleic acid-binding proteins, hormones, or protein product of a display method, such as a phage display method or a bacterial display method. More preferred polypeptide interactive sites are antibodies and enzymes. As used herein, the phrase "product of a display method" refers to any polypeptide resulting from the performance of a display method which are well known in the art. It is contemplated that any display method known in the art may be used to produce the polypeptides for use in conjunction with the present invention.

[0022] Similarly, "polynucleic acids" refer to molecules containing more than one nucleic acid. Polynucleic acids include lengths of 2 or more nucleotide monomers and encompass nucleic acids, oligonucleotides, oligos, polynucleotides, DNA, genomic DNA, mitochondrial DNA (mtDNA), copy DNA (cDNA), bacterial DNA, viral DNA, viral RNA, RNA, message RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), catalytic

RNA, clones, plasmids, M13, P1, cosmid, bacteria artificial chromosome (BAC), yeast artificial chromosome (YAC), amplified nucleic acid, amplicon, PCR product and other types of amplified nucleic acid. In preferred embodiments, the polynucleic acid may be an oligonucleotide.

[0023] Additional embodiments of detection probes of the present invention include emulsions. Particularly preferred embodiments comprise oil/water (O/W) emulsions, water/oil/water (W/O/W) emulsions, and solid/oil/water (S/O/W) emulsions, which comprise vesicles with interactive sites on their outer surfaces. The mass tag molecules are preferably encapsulated in the vesicles. In alternative embodiments, the detergents, which comprise the interface between oil and water or oil and solid phases, works as a mass tag.

[0024] Additional embodiments of detection probes of the present invention include soluble bead probes, which comprise beads with interactive sites on their outer surface. The beads preferably comprise a material that can become soluble upon physical or chemical stimulation. The mass tag molecules are preferably solidified with the material. In alternative embodiments, the bead material can preferably work as a mass tag.

[0025] Further embodiments of detection probes comprise soluble capsules comprising interactive sites on their surface. The capsules preferably comprise a material that becomes soluble upon physical or chemical stimulation. In order to release the mass tag molecules for detection, these carriers are preferably easily disrupted by physical stimulation, including but not limited to: heat, centrifugation, laser irradiation, sonication, electricity, evaporation, freeze-thaw process, or other methods known to those skilled in the art. Disruption may also be preferably achieved by chemical stimulation including, but not limited to: addition of organic solvent, detergent, acid, alkaline, enzyme, chaotropic reagents (urea, guanidium chloride, etc.), change of buffer, change of salt, change of concentration, change of pH, change of osmotic pressure, and other methods known to those skilled in the art. The mass tag molecules are preferably encapsulated within the soluble capsule. In alternative embodiments, the bead material can preferably work as a mass tag.

[0026] Additional embodiments of detection probes of the present invention include soluble porous bead probes, which comprise beads with interactive sites on their outer surface. The beads preferably comprise multiple probes, which can preferably be filled

or covered with a material that can become soluble upon physical or chemical stimulation. The mass tag molecules are preferably incorporated into the pores. In alternative embodiments, the bead material can preferably work as a mass tag.

[0027] The soluble bead probes, soluble capsule probes, and soluble porous bead probes preferably utilize a material that changes its solubility or shape upon physical (heat, centrifugation, laser irradiation, sonication, electricity, evaporation, freeze-thaw process, etc.) or chemical stimulation (addition of organic solvent, detergent, acid, alkaline, enzyme, chaotropic reagent (urea, guanidium chloride, etc.), change of buffer / salt concentration, pH, osmotic pressure, etc.). These materials works as "mass tag", or "mass tag" can be solidified, polymerized or encapsulated with them.

[0028] For example, beads or capsules made of nucleotides, peptides, saccharides or polymers can preferably become soluble by degrading these components by enzymatic or chemical reactions, or can be deformed by other means of physical or chemical stimulation. Probes made of sol-gel material (collagen, agarose, pectin, etc.) are also preferably deformed through sol-gel transformation upon heating, pH change or other forms of stimulation known to those skilled in the art. Dendrimer, sugar balls, or other forms of drug delivery carriers can also be preferably utilized for multiplex probes as such materials can typically release incorporated mass tag molecules upon stimulation.

[0029] The use of mass tag molecules in various embodiments of the present invention allows highly multiplexed assays because the mass tag molecules can be identified by their molecular mass and various analytical methods as mentioned above. Analytical methods, including but not limited to mass spectrometry, can detect even one-mass differences. For example, sixty-three fluorescent dyes of Table 1 (below) and twenty-two phospholipids of Table 2 (below) have at least five mass differences between each other, so they can be identified and quantified by mass spectrometry simultaneously.

Chemical Name	M.W.	Catalog Number	Chemical Name	M.W.	Catalog Number
7-methoxycoumarin-3-carboxylic acid	202.0	1307	WellRED D2-PA	611.0	1600
Pacific Blue	224.2	1304	DY-555	636.2	1410
7-diethylaminocoumarin-3-carboxylic acid	243.0	1306	WellRED D3-PA	645.0	1601
Marina Blue	252.3	1303	Rhodamine Red-X	654.0	1302
NBD-X	276.3	1720	DY-782	660.9	1421
Alexa Fluor 350	295.4	1730	DY-700	668.9	1417
BODIPY 493/503	302.0	1117	Alexa Fluor 568	676.8	1736
EDANS	307.1	1500	5(6)-Carboxyeosin	689.0	3310
BODIPY R6G	322.0	1106	Texas Red-X	702.0	1301
AMCA-X (Coumarin)	328.0	1300	DY-675	706.9	1416
BODIPY 564/570	348.0	1108	DY-750	713.0	1420
5-Carboxyfluorescein (FAM)	358.0	1001	DY-681	736.9	1423
BODIPY 581/591	374.0	1109	6-Hexachlorofluorescein (HEX)	744.1	1005
BODIPY FL-X	387.0	1104	LightCycler Red 705	753.0	1011
Rhodamine Green-X	394.0	1305	DY-636	760.9	1414
6-Carboxytetramethylrhodamine (TAMRA)	413.0	1202	DY-701	770.9	1424
Oregon Green 500	431.0	1102	FAR-Fuchsia (5'-Amidite)	776.0	1020
MAX	441.0	1118	DY-676	808.0	1422
Cascade Yellow	448.5	1706	Erythrosin	814.0	3311
Carboxynapthofluorescein	458.5	1725	FAR-Blue (SE)	824.0	1023
PyMPO	467.4	1710	Oyster 556	850.0	1800
JOE	487.0	1009	Oyster 656	900.0	1802
Oregon Green 514	494.0	1103	Alexa Fluor 546	964.4	1734
Cy3	508.6	1401	FAR-Green One (SE)	976.0	1024
BODIPY TR-X	519.0	1110	Alexa Fluor 660	985.0	1740
BODIPY 650/665	529.5	1107	Oyster 645	1000.0	1801
5-Fluorescein (FITC)	537.6	1000	Alexa Fluor 680	1035.0	1741
BODIPY 630/650	545.5	1113	Alexa Fluor 633	1085.0	1738
3' 6-Carboxyfluorescein (FAM)	569.5	1007	Alexa Fluor 555	1135.0	1735
Cascade Blue	580.0	1705	Alexa Fluor 750	1185.0	1743
Alexa Fluor 430	586.8	1731	Alexa Fluor 700	1285.0	1742
Lucifer Yellow	605.5	1715			

Table 1. Fluorescent Dyes (cited from Synthegen Catalog)

Carbon Number	Trivial	IUPAC	M.W.	Catalog Number
3:00	Propionoyl	Trianoic	369.4	850302
4:00	Butanoyl	Tetranoic	397.4	850303
5:00	Pentanoyl	Pentanoic	425.5	850304
6:00	Caproyl	Hexanoic	453.5	850305
7:00	Heptanoyl	Heptanoic	481.6	850306
8:00	Capryloyl	Octanoic	509.6	850315
9:00	Nonanoyl	Nonanoic	537.7	850320
10:00	Capryl	Decanoic	565.7	850325
11:00	Undecanoyl	Undecanoic	593.8	850330
12:00	Lauroyl	Dodecanoic	621.9	850335
13:00	Tridecanoyl	Tridecanoic	649.9	850340
14:00	Myristoyl	Tetradecanoic	678.0	850345
15:00	Pentadecanoyl	Pentadecanoic	706.0	850350
16:00	Palmitoyl	Hexadecanoic	734.1	850355
17:00	Heptadecanoyl	Heptadecanoic	762.2	850360
18:00	Stearoyl	Octadecanoic	790.2	850365
19:00	Nonadecanoyl	Nonadecanoic	818.2	850367
20:00	Arachidoyl	Eicosanoic	846.3	850368
21:00	Heniecosanoyl	Heneicosanoic	874.3	850370
22:00	Behenoyl	Docosanoic	902.4	850371
23:00	Trucisanoyl	Trocosanoic	930.4	850372
24:00	Lignoceroyl	Tetracosanoic	958.4	850373

Table 2. 1,2-Diacyl-sn-Glycero-3-Phosphocholine Saturated Series (Symmetric Fatty Acid) (cited from Avanti Polar Lipid Catalog)

[0030] The molecules of Tables 1 and 2 can be used as mass tags in the vesicle or vesicle components, respectively. If more probes are necessary, polynucleotides or peptides with different sequences can be utilized as mass tags or attached to mass tags because the combination of four nucleotides or twenty-one amino acids with different molecular mass can constitute hundreds of molecules with different molecular weights. This idea can be expanded to combinatorial chemistry, so hundreds, thousands, or millions of "mass tag" molecules can be prepared.

[0031] In some embodiments, the mass label may generally be any compound that may be detected by mass spectrometry. In particular embodiments, the mass label may be a biopolymer comprising monomer units, wherein each monomer unit is separately and independently selected from the group consisting essentially of an amino acid, a nucleic acid, and a saccharide with amino acids and nucleic acids being preferred monomer units. Because each monomer unit may be separately and independently selected, biopolymer mass labels may be polynucleic acids, peptides, peptide nucleic acids, oligonucleotides, and so on.

[0032] As defined herein "nucleic acids" refer to standard or naturally-occurring as well as modified/non-natural nucleic acids, often known as nucleic acid mimics. Thus, the term "nucleotides" refers to both naturally-occurring and modified/nonnaturally-occurring nucleotides, including nucleoside tri, di, and monophosphates as well as monophosphate monomers present within polynucleic acid or oligonucleotide. A nucleotide may also be a ribo; 2'-deoxy; 2', 3'-deoxy as well as a vast array of other nucleotide mimics that are well-known in the art. Mimics include chain-terminating nucleotides, such as 3'-O-methyl, halogenated base or sugar substitutions; alternative sugar structures including nonsugar, alkyl ring structures; alternative bases including inosine; deaza-modified; chi, and psi, linker-modified; mass label-modified; phosphodiester modifications or replacements including phosphorothioate, methylphosphonate, boranophosphate, amide, ester, ether; and a basic or complete internucleotide replacements, including cleavage linkages such a photocleavable nitrophenyl moieties. These modifications are well known by those of skill in the art and based on fundamental principles as described Sanger (1983), incorporated herein by reference.

[0033] Similarly, the term "amino acid" refers to naturally-occurring amino acid as well as any modified amino acid that may be synthesized or obtained by methods that are well known in the art.

[0034] In another embodiment, the mass label may be a synthetic polymer, such as polyethylene glycol, polyvinyl phenol, polyproplene glycol, polymethyl methacrylate, and derivatives thereof. Synthetic polymers may typically contain monomer units selected from the group consisting essentially of ethylene glycol, vinyl phenol, propylene glycol, methyl methacrylate, and derivatives thereof. More typically the mass label may be a polymer containing polyethylene glycol units.

[0035] The mass label is typically detectable by a method of mass spectrometry. While it is envisioned that any known mass spectometry method may be used to detect the mass labels of the present invention, methods such as matrix-assisted laser-desorption ionization mass spectrometry, direct laser-desorption ionization mass spectrometry (with no matrix), electrospray ionization mass spectrometry, secondary neutral mass spectrometry, and secondary ion mass spectrometry are preferred.

[0036] In certain embodiments the mass label has a molecular weight greater than, but not limited to, about 500 Daltons. For some embodiments, it may be preferred to have nonvolatile (including involatile) mass labels; however, for other embodiments volatile mass labels are also contemplated.

[0037] The probes of the present invention have advantages not only in multiplex capability, but also in sensitivity. According to the calculation shown in the Table 3, a 100-nm vesicle can retain 315 mass tag molecules in its inside, 62,800 molecules in its membrane, or 6,342,800 molecules in its membrane and inner vesicles. Moreover, these vesicles can preferably encapsulate more molecules by encapsulating their solid forms (powder, crystal, and other forms known to those skilled in the art) in S/O/W emulsion, soluble beads, soluble capsule. When a single detection probe retains more mass tag molecules, more sensitive detection can be accomplished. For example, Table 3 (below) indicates that a probe containing 6,342,800 mass tag molecules can increase the sensitivity $10^6 \sim 10^7$ times more than without the probe.

Diameter of Vesicle [nm]	"mass tag" molecules in a Vesicle [-]		
	Encapsulated Vesicle (*1)	Uni-lamellar Vesicle (*2)	Multi-lamellar Vesicle (*3)
1	0.00	6.28	634
10	0.32	628	63,428
100	315	62,800	6,342,800
500	39,381	1,570,000	158,570,000

Table 3. *1: Assuming that 1M "mass tag" molecules are encapsulated in vesicles. *2: Assuming that the vesicle membrane consists of 100% "mass tag" molecules and their density in the membrane is set as 0.5 nm^2/molecule (Faraday Discuss, 1998, 111, 79-94). *3: Assuming that the vesicle membrane consists of 100% "mass tag" molecules and the vesicle encapsulates 1,000,000 of 100-times smaller vesicles whose membrane also consists of 100% "mass tag" molecules.

[0038] In addition, in preferred embodiments reproducible detection can be achieved because the number of the mass tag molecules in a single probe can preferably be determined by the size of the vesicle, which can be controlled by size exclusion chromatography or membrane filtration. The size of these vesicles can be measured by

several methods, including but not limited to: size exclusion chromatography, coulter counter, light scattering, centrifugation, electron microscopy and atomic force microscopy.

[0039] These probes can preferably be applied to simultaneously analyze multiple samples (different source, different time, different stimulation, sample duplication or others known to those skilled in the art) or multiple targets (different genes, proteins, small compounds, and other targets known to those skilled in the art).

[0040] When multiple samples are to be analyzed, detection of these interactions in accordance with a preferred embodiment of the present invention can preferably be performed by the following steps: label each sample with different probes, mix the labeled samples, allow interaction with a target molecule immobilized on a surface, wash and remove unbound samples, collect the mass tag molecules from the vesicle, and quantify the mass tag molecules.

[0041] When multiple targets are to be analyzed, detection in accordance with a preferred embodiment of the present invention can preferably be performed by the following steps: combine multiple target-tethered vesicle probes, allow interaction with a sample immobilized on a surface, wash and remove unbound probes, collect the mass tag molecules from the vesicle, and quantify the mass tag molecules.

[0042] To collect the mass tag molecules, physical stimulation (including, but not limited to heat, centrifugation, laser irradiation, sonication, electricity, and other methods known to those skilled in the art) and/or chemical stimulation (including, but not limited to addition of organic solvent, detergent, acid, alkaline, chaotropic reagents, change of buffer / salt concentration, pH, osmotic pressure, and other methods known to those skilled in the art) can preferably be used to disrupt the vesicles and collect the mass tag molecules for the following analysis. Also, to analyze the "mass tag" molecules, mass spectrometry, electrophoresis or chromatography can preferably by use to identify the molecular weight (or mobility) of each mass tag. In preferred embodiments, the concentration of mass tag molecules can be simultaneously quantified. Also, these vesicle probes may preferably carry other compound tags such as raman-active compounds, fluorescent dyes and luminescent dyes

Examples

Hybridization Assay

[0043] The above encapsulated, uni-lamellar, and multi-lamellar vesicle probes were tested using a hybridization assay.

[0044] Oligo$(dA)_{20}$ and oligo$(dT)_{20}$-tethered vesicles were prepared. The encapsulated and uni-lamellar vesicles were prepared in the following steps: 20 μmol of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 20 μmol of cholesterol, 2 μmol of 1,2-dipalmitoyl-sn-glycero-3-[phosphor-rac-(1-glycerol)] (DPPG) and 1 μmol of 1,2-dipalmitoyl-sn -glycero-3-phosphoethanolamine-N-(glutaryl) (glutaryl-DPPE) were dried off in chloroform under a vacuum. The dried lipids were swelled in 1 ml of 50 mM Tris-HCl, pH 7.4, 500 mM NaCl and 100 mM sulforhodamine B (SRB) at 45°C for 1 hour. The vesicles were prepared by filtering the mixture thirty times with a 2.0-μm-pore membrane and thirty times with a 0.2-μm-pore membrane. The vesicles were purified from unincorporated SRB by G-25 column.

[0045] The multi-lamellar vesicle was prepared in several steps. For the "inside liposome," 10 μmol of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 10 μmol of cholesterol and 1.5 μmol of 1,2 -dipalmitoyl-sn-glycero-3-[phosphor-rac-(1-glycerol)] (DPPG) were dried off in chloroform under a vacuum. The dried lipids were swelled in 0.5 ml of 50 mM Tris-HCl, pH 7.4, 500 mM NaCl at 45°C for 1 hour. The "small vesicles" were prepared by sonication for 30 minutes at 45°C. The liposome encapsulated inside of the liposome was prepared by drying off 10 μmol of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 10 μmol of cholesterol, 1 μmol of 1,2-dipalmitoyl-sn-glycero-3-[phosphor-rac-(1-glycerol)] (DPPG) and 0.5 μmol of 1,2-dipalmitoyl-sn -glycero-3-phosphoethanolamine-N-(glutaryl) (glutaryl-DPPE) in chloroform under a vacuum. The dried lipids were swelled in 0.5 ml of the "inside liposome" solution (described above) at 45°C for 1 hour. The mixture was filtered 30 times with a 2.0-μm-pore membrane and 30 times with a 0.2-μm-pore membrane.

[0046] Immobilization of oligonucleotide onto the vesicles was performed in the following steps: 1 nmol oligo$(dA)_{20}$ or oligo$(dT)_{20}$ was activated with thiol modification at its 5' end by incubation in 10 mM DTT for 15 min at 45°C. The activated oligonucleotide was

purified by G-25 column. The oligonucleotide was mixed with 50 μl vesicle solution at room temperature overnight.

[0047] The mass tag of the encapsulated vesicle is sulforhodamine B (SRB) encapsulated in the vesicles, and that of the uni- and multi-lamellar vesicle was 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) that accounts for approximately 50% of the membrane components. The vesicles in the hybridization buffer (10 mM Tris, pH 7.4, 500 mM NaCl) were incubated in an oligo$(dT)_{20}$-immobilized microplate (RNAture, CA) for 1 hour at room temperature. After three washes with the hybridization buffer, the hybridized vesicles on the well surfaces were disrupted by addition of 100% methanol. The mass tag molecules were collected into methanol and were analyzed by ESI-TOF mass spectrometry (Waters, MA), and quantified by the mass intensities of the corresponding mass peaks. As shown in Figure 2, The results indicated that the vesicles with complemented oligo$(dA)_{20}$ were captured specifically in the oligo$(dT)_{20}$ microplate in comparison with those with non-complemented oligo$(dT)_{20}$ or without oligonucleotide. In Figure 2, "Oligo(dA)" and "oligo(dT)" are the oligo$(dA)_{20}$- and oligo$(dT)_{20}$-tethered vesicles captured by the oligo$(dT)_{20}$ -immobilized microplate, respectively. "W/o oligo" is the vesicle without oligonucleotide captured by the oligo$(dT)_{20}$ microplate, and "blank" is 100% methanol. In the encapsulated vesicle, the "mass tag" is sulforhodamine B and quantified by the mass peak at m/z=580.60. In the uni- and multi-lamellar vesicles, "mass tag" is DPPC and quantified by the mass peak at m/z=756.05.

Multiplex Microarray Assay

[0048] The messenger RNA or their transcribed cDNA (gene A, B, C, etc.) from Sample P1, P2, P3, etc. of Figure 3 are labeled with the mass tag probes (p1, p2, p3, etc., which correspond to P1, P2, P3, etc., respectively) to identify their respective sample sources. Labeling is acomplished by several methods such as chemical reaction with active residues on the probe, incorporation of the dNTP attached with the probes and cDNA synthesis using the oligo(dT) or specific sequence primer tethered on the probes. These labeled genes are combined together and applied to a surface with multiple spots where specific sequence polynucleotides (gene a, b, c, etc., which are complement with gene A, B, C, etc.,

respectively) are immobilized. After hybridization and several washes, each gene spot captures its respective complement gene from multiple samples. Laser irradiation onto each spot disrupts the probes on the spot and ionizes the mass tag molecules for mass spectrometry (MALDI, etc.). Alternatively, addition of organic solvent disrupts the probes and collects the mass tag molecules for mass spectrometry (ESI, etc.) or other analytical methods. The mass peaks of the mass tag molecules obtained from each gene spot simultaneously give the amounts of its complement gene expressed in different samples.

Multiplex Immunoassay

[0049] Antibodies (A, B, C, etc.) from sample P1, P2, P3, etc. of Figure 4 are labeled with the mass tag probes (p1, p2, p3, etc., which corresponds to P1, P2, P3, etc., respectively). The labeled antibodies are combined together and applied to a microplate where each well has a different antigen (a, b, c, etc., which specifically bind to Antibody A, B, C, etc., respectively). After incubation and several washes, each well captures its respective antibody from multiple samples. Laser irradiation onto each well disrupts the probes on the spot and ionizes the mass tag molecules for mass spectrometry (MALDI, etc.) or addition of organic solvent disrupts the probes and collects the mass tag molecules for mass spectrometry (ESI, etc.). The mass peaks of the mass tag molecules obtained from each well simultaneously give the amounts of the corresponding antibody expressed in different samples.

Multiplex Hybridization Assay

[0050] Multiple hybridization probes (Probe P1, P2, P3, etc.) tethered with their corresponding sequence-specific oligonucleotides (Oligo A, B, C, etc., which are complement to target genes, Gene a, b, c, etc., respectively) are prepared, as illustrated in Figure 4. These probes are combined together and applied to a sample DNA or RNA immobilized on a solid surface by hybridization (mRNA captured by oligo(dT)-coated surface, etc.), physical adsorption (DNA/RNA captured on a glass-fiber surface, etc.), synthesis (DNA/RNA synthesis by polymerase or chemical reaction, etc.) or other methods known to those skilled in the art. After hybridization and several washes, only the probes

corresponding to the genes expressed in the sample are captured on the surface. Therefore, analyzing the concentrations of the mass tag molecules on the surface simultaneously gives the multiple gene profiles in the sample DNA/RNA.

Multiplex CpG Methylation Assay

[0051] Multiple oligonucleotide-tethered probes are prepared to hybridize against specific sequences of their corresponding CpG methylation sites on genomic DNA. These probes are combined together and applied to fragmented sample DNA captured on a surface coated with CpG-methyl-specific antibody or chemical residue. After incubation and several washes, only the probes corresponding to the CpG methyls expressed in the sample are captured on the surface. Therefore, analyzing the concentrations of the "mass tag" molecules on the surface gives the expression profiles of multiple CpG methylation sites in the sample simultaneously.

Capillary Assay

[0052] As illustrated in Figure 6, biological samples (DNA, RNA, protein, small compound, etc.) labeled with the probes or target-specific probes (hybridization probe, antibody probe, etc.) are prepared, combined and applied to a capillary. On the inside wall of the capillary, specific target molecules or samples (DNA, RNA, protein, small compound, etc.) are immobilized. After incubation and several washes, specifically bound molecules are captured on the inside wall of the capillary by intermolecular interaction such as hybridization, protein-protein interaction or antigen-antibody interaction. Limited amount of organic solvent disrupts the probe on the wall and collects the "mass tag" molecules in the limited volume when it passes through the capillary, therefore the collected "mass tag" molecules are analyzed at higher concentration.

Figure 1 A. Encapsulated Vesicle Type

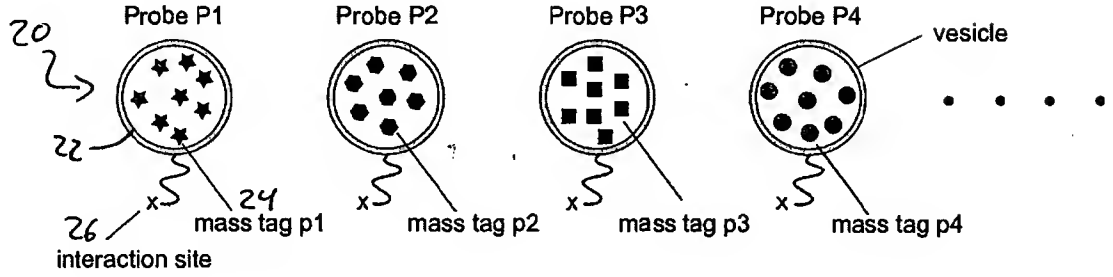


Figure 1B. Uni-lamellar Vesicle Type

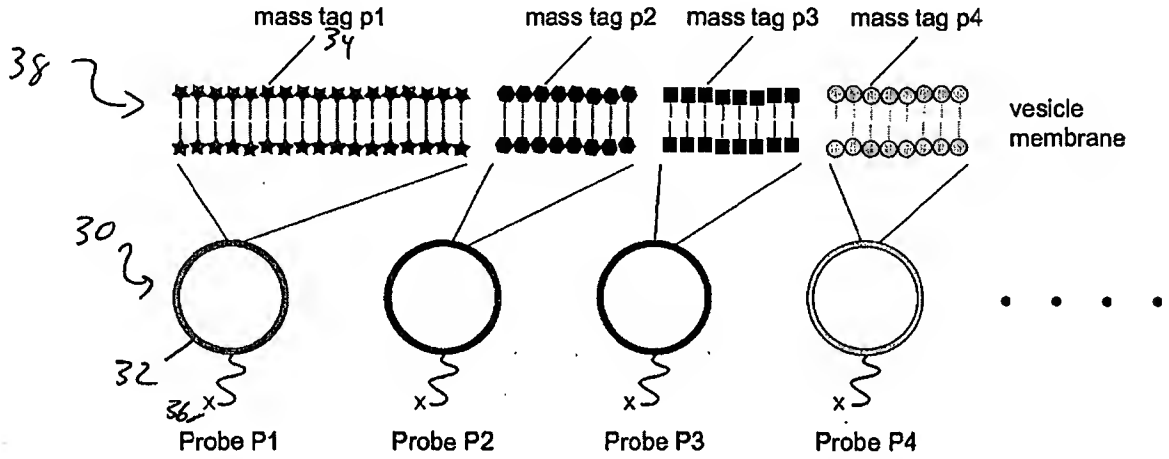
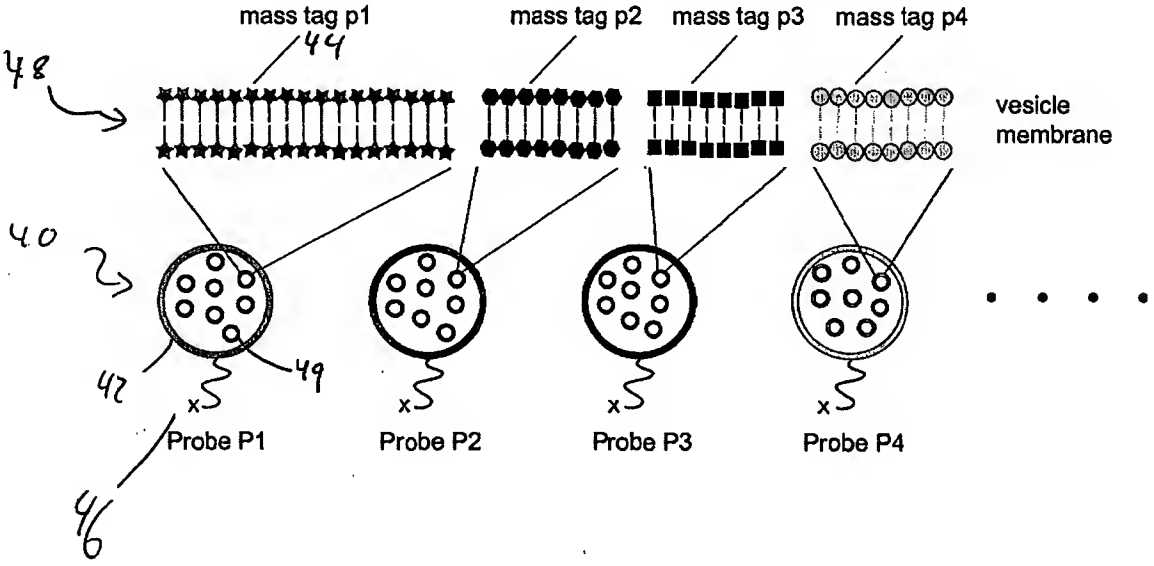


Figure 1 C. Multi-lamellar Vesicle Type



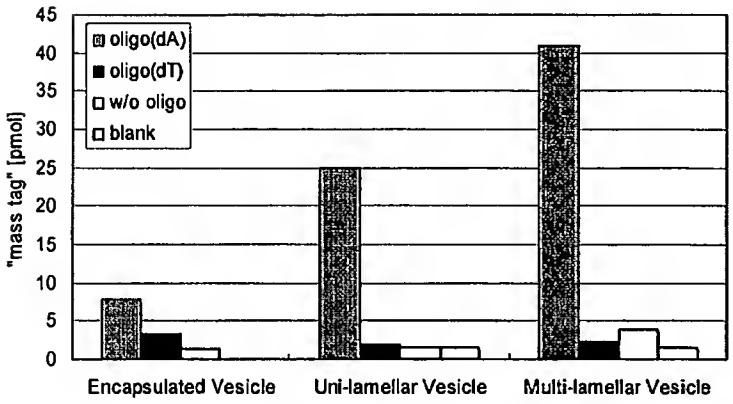

45
40
35
30
25
20
15
10
5
0
"mass tag" [pmol]
oligo(dA)
oligo(dT)
w/o oligo
blank
Encapsulated Vesicle
Uni-lamellar Vesicle
Multi-lamellar Vesicle

Figure 2

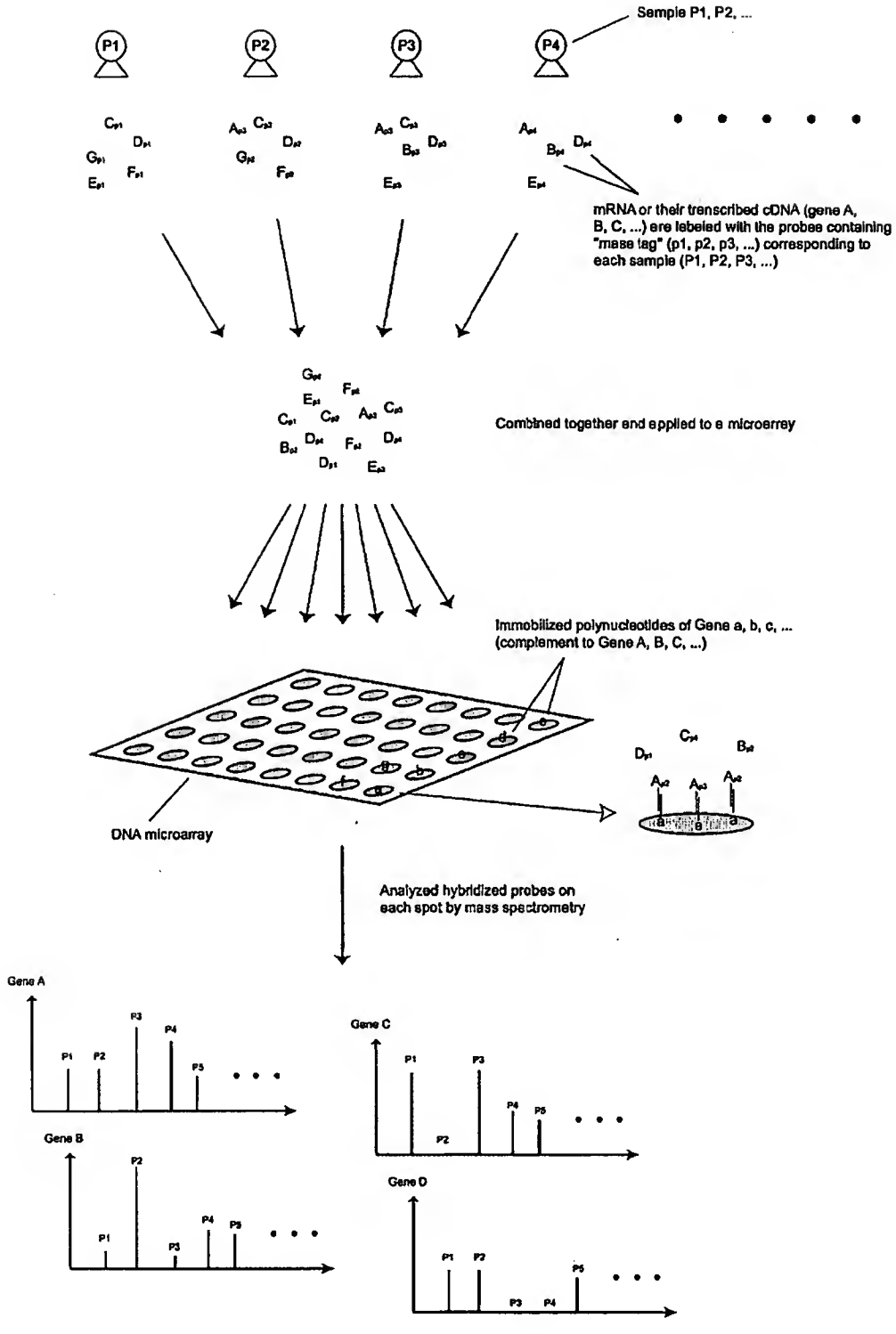

Sample P1, P2, ...
P1
P2
P3
P4
mRNA or their transcribed cDNA (gene A, B, C, ...) are labeled with the probes containing "mass tag" (p1, p2, p3, ...) corresponding to each sample (P1, P2, P3, ...)
Combined together and applied to a microarray
Immobilized polynucleotides of Gene a, b, c, ... (complement to Gene A, B, C, ...)
DNA microarray
Analyzed hybridized probes on each spot by mass spectrometry
Gene A
Gene B
Gene C
Gene D

Figure 3

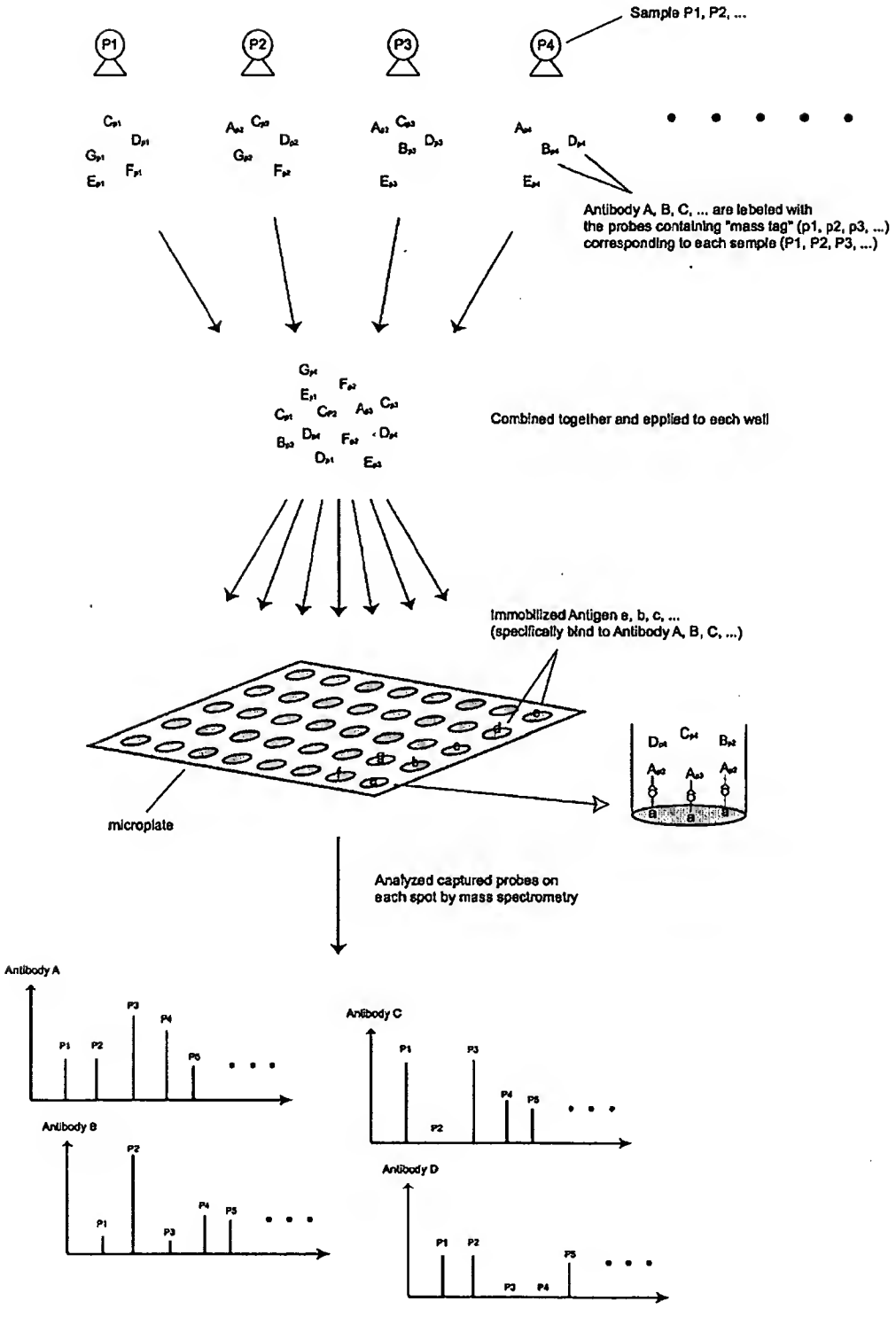

Sample P1, P2, ...
P1
P2
P3
P4
Antibody A, B, C, ... are labeled with
the probes containing "mass tag" (p1, p2, p3, ...)
corresponding to each sample (P1, P2, P3, ...)
Combined together and applied to each well
Immobilized Antigen a, b, c, ...
(specifically bind to Antibody A, B, C, ...)
microplate
Analyzed captured probes on
each spot by mass spectrometry
Antibody A
Antibody B
Antibody C
Antibody D

Figure 4

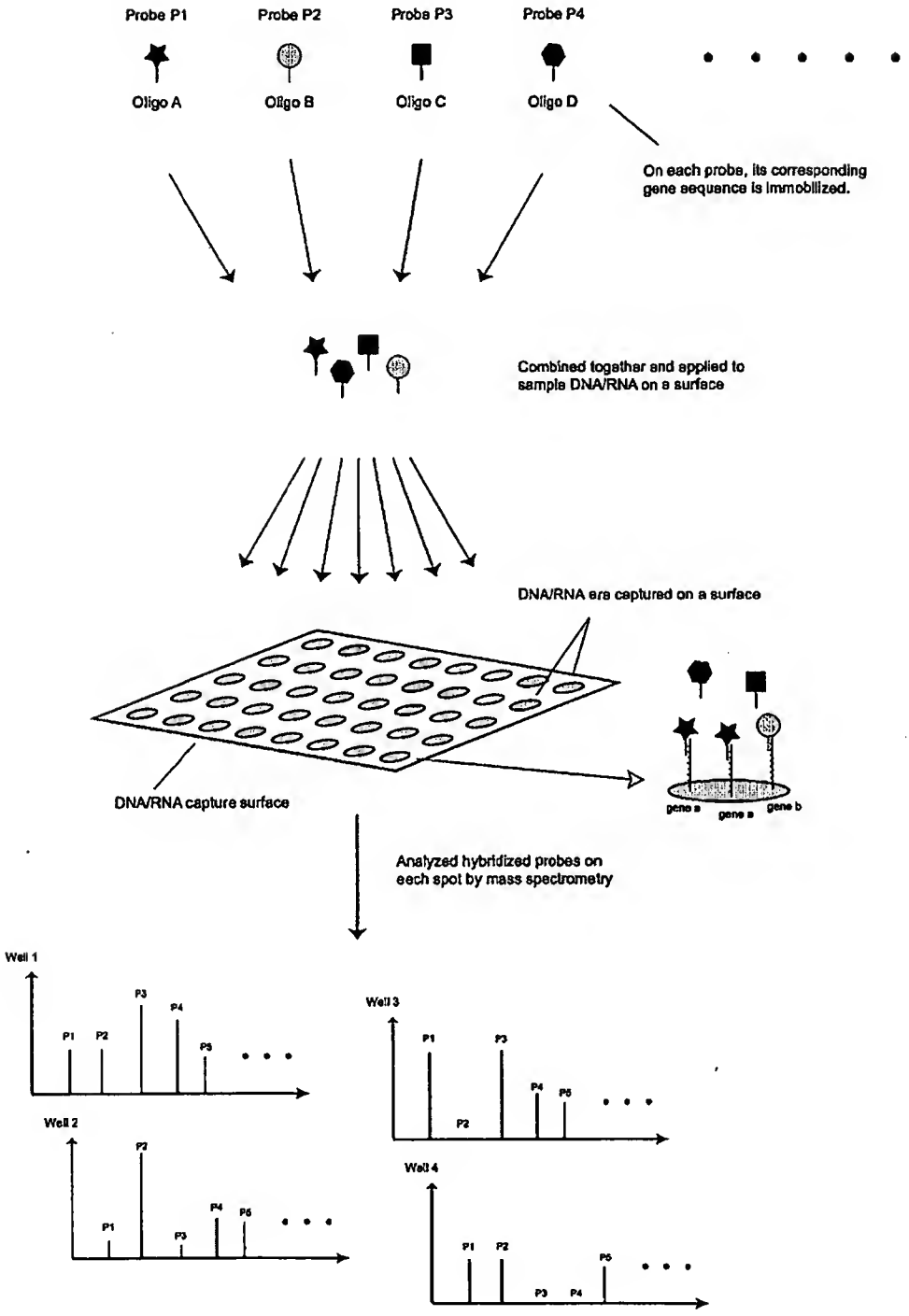

Probe P1
Probe P2
Probe P3
Probe P4
Oligo A
Oligo B
Oligo C
Oligo D
On each probe, its corresponding gene sequence is immobilized.
Combined together and applied to sample DNA/RNA on a surface
DNA/RNA are captured on a surface
DNA/RNA capture surface
gene a
gene a
gene b
Analyzed hybridized probes on each spot by mass spectrometry
Well 1
Well 2
Well 3
Well 4

Figure 5

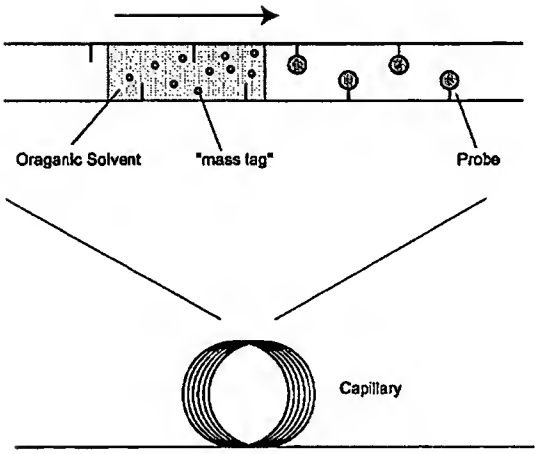


Figure 6